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Ebola Virus Protein VP35 Impairs the Function of Interferon Regulatory Factor-Activating Kinases IKKε and TBK-1[▽]

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The Ebola virus (EBOV) VP35 protein antagonizes the early antiviral alpha/beta interferon (IFN-α/β) response. We previously demonstrated that VP35 inhibits the virus-induced activation of the IFN-β promoter by blocking the phosphorylation of IFN-regulatory factor 3 (IRF-3), a transcription factor that is crucial for the induction of IFN-α/β expression. Furthermore, VP35 blocks IFN-β promoter activation induced by any of several components of the retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA-5)-activated signaling pathways including RIG-I, IFN-β promoter stimulator 1 (IPS-1), TANK-binding kinase 1 (TBK-1), and IκB kinase epsilon (IKKε). These results suggested that VP35 may target the IRF kinases TBK-1 and IKKε. Coimmunoprecipitation experiments now demonstrate physical interactions of VP35 with IKKε and TBK-1, and the use of an IKKε deletion construct further demonstrates that the amino-terminal kinase domain of IKKε is sufficient for interactions with either IRF-3 or VP35. In vitro, either IKKε or TBK-1 phosphorylates not only IRF-3 but also VP35. Moreover, VP35 overexpression impairs IKKε–IRF-3, IKKε–IRF-7, and IKKε–IPS-1 interactions. Finally, lysates from cells overexpressing IKKε contain kinase activity that can phosphorylate IRF-3 in vitro. When VP35 is expressed in the IKKε-expressing cells, this kinase activity is suppressed. These data suggest that VP35 exerts its IFN-antagonist function, at least in part, by blocking necessary interactions between the kinases IKKε and TBK-1 and their normal interaction partners, including their substrates, IRF-3 and IRF-7.

Ebola viruses (EBOVs), members of the family *Filoviridae*, are filamentous, enveloped, negative-sense, single-stranded RNA viruses which cause frequently lethal hemorrhagic fevers in humans and nonhuman primates (44). EBOV disease is characterized by fever, shock, coagulation defects, and impaired immunity. Fatal infections are also characterized by progressively increasing systemic viral titers and cytokines, consistent with a model in which host innate and adaptive immune responses are unable to control infection, while the inflammatory response becomes overactivated, causing disease (5, 12).

EBOV infection blocks cellular alpha/beta interferon (IFN-α/β) responses; critical components of the host innate immune response to virus infection (13–15, 23). Two EBOV proteins appear to function in the suppression of IFN-α/β responses, VP35 and VP24 (2, 3, 7, 9, 16, 18, 19, 41, 42). The VP35 protein is a multifunctional protein that plays a key role in viral replication and nucleocapsid assembly (22, 35, 36). VP35 possesses a carboxy-terminal domain with a unique fold that allows double-stranded RNA (dsRNA) binding, a function that may be necessary for the inhibition of IFN-α/β production (2, 3, 7, 9, 28). The VP24 protein impairs cellular responses to exogenous IFN-α/β and IFN-γ by blocking the nuclear import of activated STAT1 (41, 42). Recently, a mutation of individual basic amino acids within the carboxy terminus of VP35 rendered recombinant EBOVs less able to inhibit IFN-α/β responses in cell culture, resulted in an enhanced activation of IFN-regulatory factor 3 (IRF-3), and attenuated the virus in cell culture and in vivo (16–18). Because VP35 contributes to virus escape from host innate immunity and is required for virulence, an understanding of the mechanisms by which it acts as an IFN antagonist is of importance.

In cells, IFN-α/β activates an antiviral state which can limit spread of infection and which also influences adaptive immune responses (11). Upon virus infection, the IFN-α/β response can be triggered by cytoplasmic sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) (24, 53, 54). Signal propagation occurs through the mitochondrion-associated adapter IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, or CARDIF) (25, 33, 45, 52), which subsequently activates inhibitor of κB kinase epsilon (IKKε) and TANK-binding kinase 1 (TBK-1) (10, 20, 25, 32, 33, 46). These kinases in turn phosphorylate the otherwise inactive transcription factor IRF-3 and/or IRF-7. Phosphorylation results in activation, dimerization, and translocation to the nucleus, where IRF-3 and/or IRF-7 contribute to the transcription of IFN-α/β genes (26, 30, 55). In most cell types, constitutively expressed IRF-3 is activated predominately during the initial response to virus infection. This triggers the expression of IFN-β and select IFN-α genes. IRF-7 activates a larger number of IFN-α genes, and its expression is IFN inducible. Thus, the induction of IRF-7 expression and its subsequent activation provide a means of amplification of the IFN-α/β response (29).

VP35 expression prevents the phosphorylation, dimerization, and nuclear translocation of IRF-3 induced by virus infection, thereby inhibiting IFN-α/β gene expression (2).

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Furthermore, VP35 can block the activation of the IFN-β promoter induced by the overexpression of any of several components of the RIG-I signaling pathway, including RIG-I, IPS-1, IKKε, or TBK-1 (7). However, VP35 does not prevent IFN-β promoter activation induced by a constitutively active IRF-3 (IRF-3 5D) (2, 7). These data suggest that VP35 may act proximal to the IRF-3 and IRF-7 kinase IKKε or TBK-1 to suppress IFN-α/β gene expression.

In this report, we provide evidence that VP35 physically interacts with IKKε and TBK-1 and is phosphorylated by these kinases. Moreover, we show that VP35 can impair IKKε–IRF-3, IKKε–IRF-7, and IKKε–IPS-1 interactions. Consistent with a model in which VP35 targets the IRF-3 kinases, the kinase activity of lysates from cells transfected with IKKε is decreased when VP35 is present.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies against the Zaire EBOV VP35 (6C5) and Zaire EBOV nucleoprotein (NP) were generated in collaboration with the Mount Sinai Hybridoma Center. The monoclonal anti-hemagglutinin (HA) and anti-FLAG (M2) and polyclonal anti-HA and anti-FLAG antibodies were purchased from Sigma (St. Louis, MO).

Cell lines and viruses. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Sendai virus (SeV) strain Cantell was grown in 10-day-old embryonated chicken eggs for 2 days at 37°C.

Plasmids. Zaire EBOV VP35, FLAG–RIG-I, and IPS-1 were cloned into pCAGGS as described elsewhere previously (3, 7, 37). Plasmids encoding human cDNAs for wild-type TBK-1 and IKKε were kindly provided by John Hiscott (McGill University). FLAG-tagged versions of these cDNAs were amplified by PCR and inserted into expression plasmid pCAGGS (37). A kinase-inactive IKKε (IKKεKN) was generated by introducing the previously described K38A mutation into IKKε (39, 40, 47). A kinase-inactive K38M mutant of TBK-1 (TBK-1KN) was kindly provided by Benjamin tenOever (Mount Sinai School of Medicine). Plasmids encoding human IRF-3 were previously described (2). IRF-3 amino acids 375 to 427 were amplified by PCR for expression as glutathione *S*-transferase (GST) fusions in *Escherichia coli*. The pCAGGS-FLAG-IRF-7 construct was kindly provided by Adolfo García-Sastre (Mount Sinai School of Medicine).

Bacterial expression and purification of GST and the GST–IRF-3 C terminus. GST and the GST–IRF-3 C terminus, residues 375 to 427 (IRF-3-C), were expressed in *E. coli* Origami B BL21(DE3)pLysS host strains (Stratagene). Cultures were grown at 37°C to an optical density at 600 nm of 0.57, and IRF-3 expression was induced by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Induced cells were grown further at 18°C for 24 h. Lysates were prepared by sonication for 10 s five times in lysis buffer (25 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.1 mM dithiothreitol, and a cocktail of protease inhibitors [Roche]). Bacterially produced protein was purified from cell lysates on a glutathione-Sepharose (Amersham Biosciences) column. After loading, the column was washed with a solution containing 25 mM Tris-HCl, 1 M NaCl, 0.1% NP-40, and 1 mM EDTA and eluted with a solution containing 5 mM glutathione, 25 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM Tris(2-carboxyethyl)phosphine, 5% glycerol, and 0.2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}. Dialysis was then performed overnight in 1 liter of kinase buffer (20 mM HEPES, 1 mM beta-glycerophosphate, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM $NaVO_3$).

Transfections. HEK 293T cells were transfected with a 1:1 ratio of Lipofectamine 2000 to plasmid DNA in OptiMEM medium (Gibco) at 37°C for 8 h. For subsequent infection of cells, the transfection medium was removed, and SeV was added, at a multiplicity of infection of 10, in a solution containing phosphate-buffered saline and 0.3% bovine serum albumin for 1 h. Infection medium was then replaced with Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and cells were incubated at 37°C overnight. Following overnight incubation, cells were lysed in lysis buffer (50 mM Tris [pH 8], 1% NP-40, 280 mM NaCl, 0.2 mM EDTA, 2 mM EGTA, and 10% glycerol).

Immunoprecipitations. Lysates were incubated with 1 μg of the indicated antibody for 4 h at 4°C, followed by a 1-h incubation with protein A-Sepharose beads (Roche). Beads were washed five times with lysis buffer. After washing, beads were resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer, separated by 10% SDS-PAGE, and analyzed by Western blotting as indicated.

Purification of FLAG-tagged proteins. HEK 293T cells were transfected with 2 μg of expression plasmids for FLAG-tagged IKKε, IKKεKN, TBK-1, TBK-1KN, or VP35. The transfected cell lysates were immunoprecipitated with M2 anti-FLAG affinity gel (Sigma). The FLAG-tagged proteins were eluted from the affinity gel by two sequential incubations with FLAG peptide at 100 μg/ml. The eluate was concentrated 20-fold, and buffer was exchanged to kinase buffer with a Microcon centrifugal filter device (Millipore). Proteins were stored at −80°C in kinase buffer (without dithiothreitol or $NaVO_3$) supplemented with 4% glycerol.

In vitro kinase assays. Purified FLAG-tagged IKKε, TBK-1, TBK-1KN, or IKKεKN was incubated with a solution containing 5 μCi [γ-^{32}P]ATP (Perkin-Elmer); 0.1 mM unlabeled ATP; and either GST, GST–IRF-3 (residues 375 to 427), or FLAG-tagged VP35 in 30 μl kinase buffer (20 mM HEPES, 1 mM beta-glycerophosphate, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM $NaVO_3$) as described previously (27). Reaction mixtures were incubated at 30°C for 30 min and terminated by the addition of SDS sample loading buffer. Proteins were separated by 12% SDS-PAGE, and phosphorylation was visualized by autoradiography.

To examine IRF-3 phosphorylation by cell lysates, in vitro kinase assays were performed as described above using GST–IRF-3-C as a substrate and lysates from 12% of 2×10^6 cells (2.4×10^5 cell equivalents) cotransfected with FLAG-IKKε expression plasmid and either empty vector as a source of kinase, increasing concentrations of VP35 expression plasmid (1 μg, 2 μg, and 4 μg), or EBOV NP plasmid. Kinase reactions were terminated after 1 h by the addition of glutathione-Sepharose (Amersham Biosciences) in lysis buffer for affinity purification of GST–IRF-3-C. Following incubation on a Nutator mixer for 1 h, beads were washed five times with lysis buffer. SDS-PAGE sample loading buffer was added, and proteins were separated by 10% or 12% SDS-PAGE. Phosphorylation was visualized by autoradiography and quantified by ImageJ software. Transfected proteins were visualized by Western blotting of lysates from 1×10^5 cell equivalents with the indicated antibodies.

Reporter assay. 293T cells were transfected with the indicated amount of expression plasmid DNA together with 400 ng of the IFN-α4–chloramphenicol acetyltransferase (CAT) reporter plasmid (2) and 200 ng of the constitutive firefly luciferase reporter plasmid. Twelve hours posttransfection, cells were infected with SeV or mock infected for 1 h. Twelve hours postinfection, cells were lysed with reporter lysis buffer (Promega), and CAT activities were measured (43). Firefly luciferase activity was determined as recommended by the manufacturer (Promega) and was used to normalize CAT activity. IFN-α4 reporter gene activation is expressed as induction over an empty vector mock-infected control.

RESULTS

VP35 interacts with the IRF-3 kinases IKKε and TBK-1. VP35 was previously reported to block the phosphorylation of IRF-3 and to inhibit the IRF-3-dependent gene expression induced by the overexpression of IKKε and TBK-1 (2, 7). To test whether IKKε and TBK-1 are targeted by VP35, coimmunoprecipitation (co-IP) experiments were performed. 293T cells were transfected with expression plasmids for FLAG-tagged kinase-inactive forms of IKKε (IKKεKN) (Fig. 1, lanes 2, 5, 7, 8, and 10) or TBK-1 (TBK-1KN) (Fig. 1, lanes 1, 4, and 6) (39, 40, 47) alone or with VP35 (Fig. 1, lanes 3 to 7, 9, and 10). Inactive kinases were used for co-IP experiments because the interaction of the kinases with their substrate IRF-3 is more readily detected by co-IP when inactive rather than functional kinases are used (data not shown). This presumably reflects the fact that active kinases, upon overexpression, rapidly phosphorylate IRF-3, resulting in its nuclear accumulation. The VP35-kinase interactions, however, can be detected by co-IP with equal efficiency using either the kinase-active or -inactive forms (data not shown). Twelve hours posttransfection, cells were either mock infected (Fig. 1, lanes 1 to 5) or infected with SeV (lanes 6 to 10). Twenty-four hours posttransfection, cells were lysed as described in Materials and Meth-

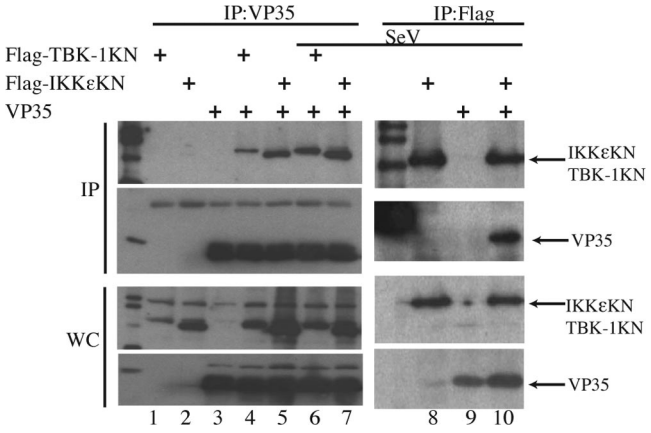

FIG. 1. IKKε and TBK-1 coprecipitate with VP35. 293T cells were transfected with expression plasmids encoding the indicated proteins (TBK-1KN [lanes 1, 4, and 6], IKKεKN [lanes 2, 5, 7, 8, and 10], and VP35 [lanes 3 to 7, 9, and 10]). Twelve hours posttransfection, cells were left uninfected (lanes 1 to 5) or were infected with SeV strain Cantell for 1 h (lanes 6 to 10). Twelve hours later, cells were harvested and lysed. The precleared lysates were immunoprecipitated (IP) using either monoclonal anti-VP35 (IP:VP35) (lanes 1 to 7) or monoclonal anti-FLAG antibody (IP:FLAG). After SDS-PAGE, Western blotting was performed using monoclonal anti-VP35 or anti-FLAG antibody. The expression of VP35 and FLAG-tagged kinase constructs was confirmed by Western blot analysis of whole-cell lysates (WC) with anti-VP35 and anti-FLAG antibodies (bottom).

ods. Immunoprecipitations were then performed on lysates by using an anti-VP35 monoclonal antibody (Fig. 1, lanes 1 to 7). IKKεKN and TBK-1KN each coprecipitated with VP35 (lanes 4 to 7). The reciprocal co-IP was also performed with FLAG-IKKεKN using anti-FLAG monoclonal antibodies (lanes 8 to 10). When an anti-FLAG immunoprecipitation was performed, VP35 coprecipitated with FLAG-IKKεKN (Fig. 1, lane 10). Therefore, VP35 interacts with the IRF kinases.

IKKεKN and TBK-1 can phosphorylate VP35 in vitro. Having demonstrated a physical interaction between VP35 and both IKKε and TBK-1, we sought to determine whether IKKε or TBK-1 can phosphorylate VP35 in an in vitro kinase assay. FLAG-tagged IKKε, FLAG-tagged TBK-1, and FLAG-tagged VP35 were each purified from separate, transiently transfected 293T cell cultures. Increasing amounts of FLAG-IKKε (Fig. 2A and B) were incubated with constant amounts of GST (Fig. 2A and B, lanes 1 to 3), GST fused to the C-terminal region of IRF-3 (amino acids 375 to 427) (IRF-3-C) (Fig. 2A and B, lanes 4 to 6), or FLAG-VP35 (Fig. 2A and B, lanes 7 to 9). In vitro kinase assays were performed as described in Materials and Methods. The products were separated by SDS-PAGE and developed by autoradiography (Fig. 2A) and by Coomassie blue staining (Fig. 2B). Alternatively, FLAG–TBK-1 was used in in vitro kinase assays (Fig. 2C and D, lanes 4 to 7) with GST (lanes 1 and 5), GST–IRF-3-C (lanes 2 and 6), or FLAG-VP35 (lanes 3 and 7).

As previously reported, both IKKε and TBK-1 undergo ap-

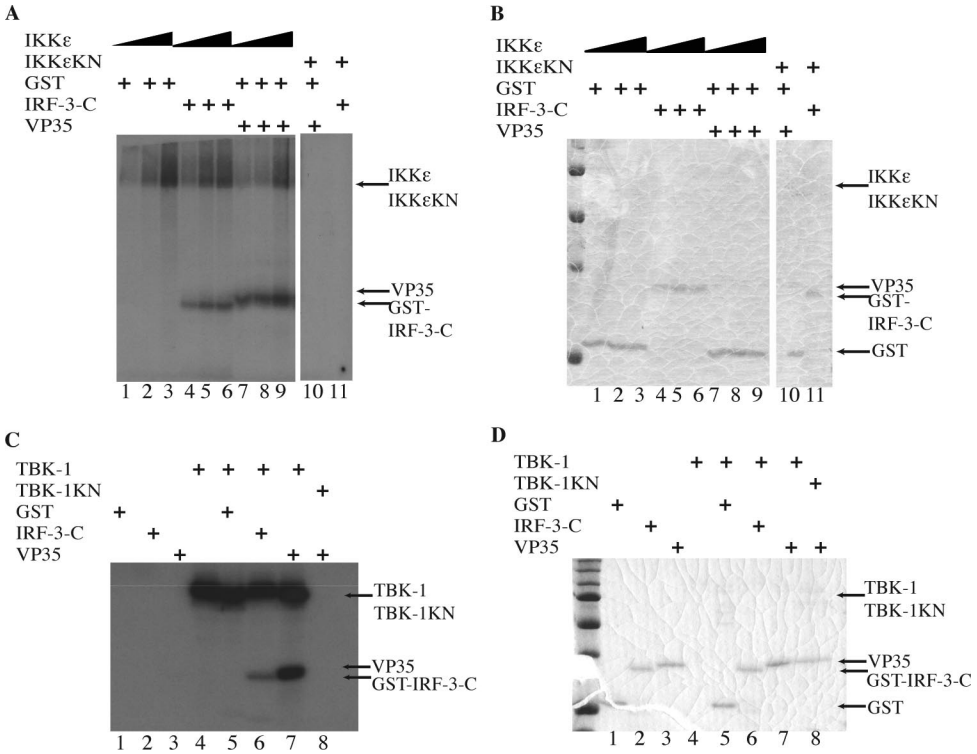

FIG. 2. IKKε and TBK-1 can directly phosphorylate VP35 by in vitro kinase assay. In vitro kinase assays were performed with FLAG-tagged kinase, GST, GST–IRF-3-C (amino acids 375 to 427), or FLAG-VP35 serving as a substrate. Proteins were separated by SDS-PAGE, phosphorylation was visualized by autoradiography (A and C), and proteins were visualized by Coomassie blue staining (B and D). FLAG-IKKε was used in A and B (lanes 1 to 9), and TBK-1 was used in C and D (lanes 4 to 6). The kinase-inactive forms were used in A and B (lanes 10 and 11) and in C and D (lane 8).

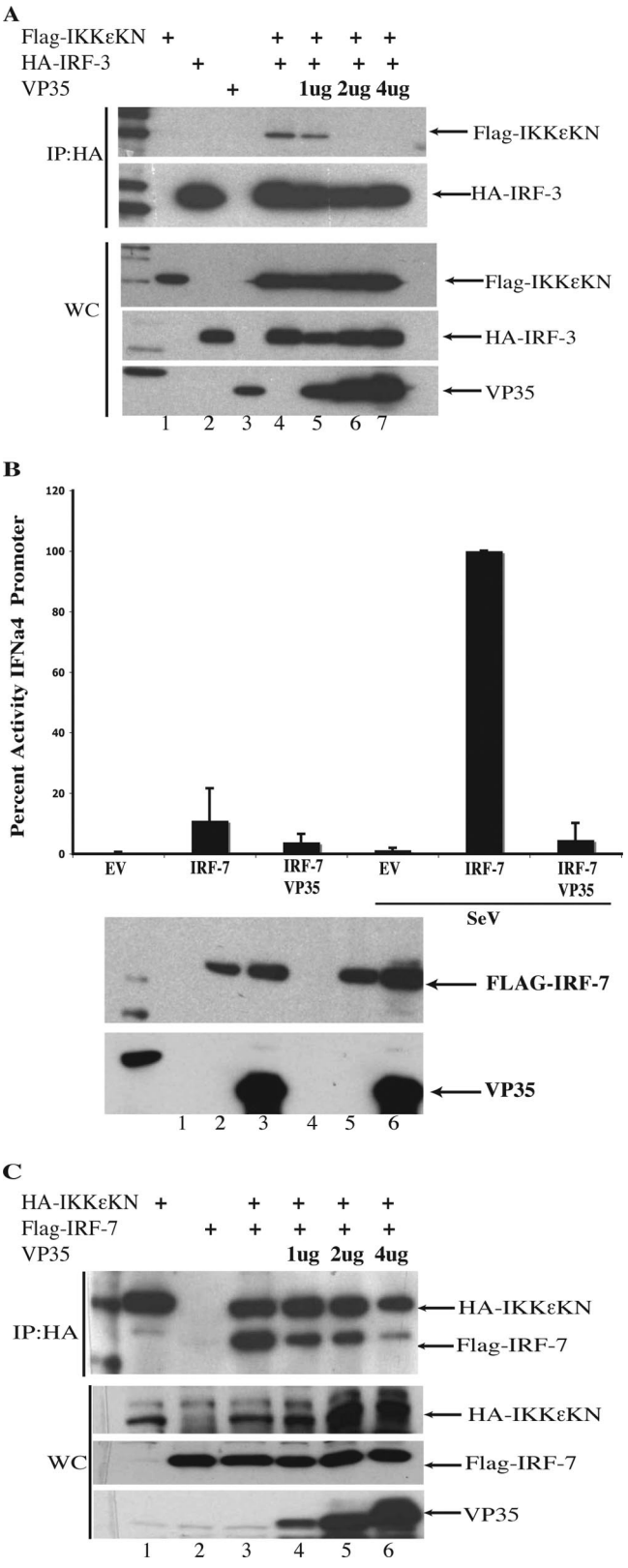

FIG. 3. VP35 disrupts IKKε interactions with IRF-3 and IRF-7. (A) FLAG-tagged kinase-inactive IKKε (FLAG-IKKεKN) and HA-tagged IRF-3 and VP35 expression plasmids were transfected alone (lanes 1 to 3, respectively), or FLAG-IKKεKN and HA–IRF-3 were cotransfected in the absence (lane 4) or presence (lanes 5 to 7) of increasing concentrations of VP35 expression plasmid. HA–IRF-3 was then precipitated with anti-HA antibody, and the resulting pellets were probed with anti-FLAG and anti-HA antibody to detect IKKεKN or IRF-3, respectively. Levels of FLAG-IKKεKN, HA–IRF-3, and VP35 in whole-cell extracts (WC) are also shown. (B) 293T cells were transfected with expression plasmids encoding the IFN-α4–CAT reporter gene; a constitutive firefly luciferase reporter; and either empty vector (samples 1 and 4), FLAG-tagged IRF-7 alone (samples 2 and 5), or FLAG-tagged IRF-7 with VP35 (samples 3 and 6). Twelve hours posttransfection, cells were either mock infected or infected with SeV, as indicated. Twelve hours postinfection, cells were harvested, and CAT and luciferase activities were determined. Values are expressed as induction over empty-vector mock-infected control. Virus-induced CAT activity was normalized to firefly luciferase activity. Error bars indicate standard deviations. Expression levels of IRF-7 and VP35 were determined by Western blotting (inset). Blots were probed with a monoclonal antibody to VP35 and a monoclonal antibody to FLAG. (C) HA-tagged kinase-inactive IKKε (HA-IKKεKN) and FLAG–IRF-7 expression plasmids were transfected alone (lanes 1 and 2), or HA-IKKεKN and FLAG–IRF-7 were cotransfected in the absence (lane 3) or presence (lanes 4 to 6) of increasing concentrations of VP35 expression plasmid. HA-IKKεKN was then precipitated with anti-HA antibody, and the resulting pellets were probed with anti-FLAG and -HA antibodies. Levels of FLAG–IRF-7, HA-IKKεKN, and VP35 in whole-cell extracts (WC) are also shown. IP, immunoprecipitation.

parent autophosphorylation (Fig. 2A and C) (47). Neither kinase phosphorylated the negative control protein GST (Fig. 2A, lanes 1 to 3, and C, lane 5), nor was VP35 or GST–IRF-3-C phosphorylated when the kinase-negative forms of the kinases were used (Fig. 2A, lanes 10 to 11, and C, lane 8). The kinase-competent IKKε phosphorylated GST–IRF-3-C as well as VP35, shown in Fig. 2A, lanes 4 to 6 and 7 to 9, respectively. TBK-1 also phosphorylated GST–IRF-3-C and VP35, as shown in Fig. 2C, lanes 6 and 7, respectively. The examination of the Coomassie blue-stained gels demonstrated that the purified protein preparations did not contain visible amounts of contaminating cellular proteins and that comparable amounts of GST, GST–IRF-3-C, and VP35 were present in the kinase reactions (Fig. 2B and D). These data demonstrate that IKKε and TBK-1 can phosphorylate VP35.

VP35 disrupts IKKεKN–IRF-3 and IKKεKN–IRF-7 interactions. The interaction of VP35 with IKKε and TBK-1, coupled with the phosphorylation of VP35 by these kinases, suggested that VP35 might act as an alternative substrate which blocks the interaction between IKKε or TBK-1 and their IRF substrates. To address this question, co-IP experiments were performed, focusing on IKKε as a representative IRF-3 kinase. Cells were cotransfected with full-length FLAG-IKKεKN (Fig. 3A, lanes 1 and 4 to 7) and HA–IRF-3 (lanes 2 and 4 to 7) plasmids in the absence (lane 4) or the presence (lanes 5 to 7) of increasing amounts of VP35 plasmid. IRF-3 was immunoprecipitated using monoclonal anti-HA antibody, and coprecipitated FLAG-IKKεKN was analyzed by Western blotting with anti-FLAG polyclonal antibody. HA–IRF-3 pulled down FLAG-IKKεKN, and the amount of IKKεKN that coprecipitated with IRF-3 decreased as the amount of VP35 increased (Fig. 3A, top).

VP35 was previously reported to block IRF-3-dependent gene expression induced by SeV infection (2, 7). To determine whether VP35 could block IRF-7-dependent gene expression as well, reporter assays were performed utilizing an IRF-7-

dependent promoter, IFN-α4. 293T cells were transfected with an IFN-α4–CAT reporter plasmid, a constitutive firefly luciferase plasmid, and either empty vector (Fig. 3B, samples 1 and 4), FLAG–IRF-7 alone (samples 2 and 5), or FLAG–IRF-7 with VP35 expression plasmids (samples 3 and 6). Cells were subsequently mock infected or infected with SeV at a multiplicity of infection of 10. The expression of IRF-7 alone was sufficient to weakly induce IFN-α4 promoter activation relative to empty vector-transfected cells (Fig. 3B, samples 1 and 2). This IRF-7-induced activation of the promoter was decreased upon coexpression with 3 μg of VP35 (sample 3). SeV infection of IRF-7-expressing cells resulted in a dramatic induction of the IFN-α4 reporter (sample 5) compared with that seen for mock-infected cells (samples 1 and 2) and SeV-infected empty vector-expressing cells (sample 4), highlighting the role of IRF-7 in this reporter activation. The coexpression of VP35 with IRF-7 drastically decreased SeV-induced IFN-α4 reporter activation (sample 6). Therefore, VP35 is able to block the SeV-mediated activation of an IRF-7-dependent promoter.

Consistent with this functional assay, a biochemical assay similar to that shown in Fig. 3A was performed. 293T cells were cotransfected with full-length HA-IKKεKN (Fig. 3C, lanes 1 and 3 to 6) and FLAG–IRF-7 (lanes 2 to 6) plasmids in the absence (lane 3) or the presence (lanes 4 to 6) of increasing amounts of VP35 plasmid. IKKεKN was immunoprecipitated using a monoclonal anti-HA antibody, and coprecipitated FLAG-IRF-7 was analyzed by Western blotting with anti-FLAG polyclonal antibody. HA-IKKεKN pulled down FLAG–IRF-7 (Fig. 3C, lane 3), and the amount of IRF-7 that coprecipitated with IKKεKN decreased when increasing amounts of VP35 were present. The loss of the IRF-7–kinase interaction was most dramatic when the highest concentration of VP35 was present (Fig. 3C, lane 6). Notably, in both the IRF-3 (Fig. 3A) and IRF-7 (Fig. 3C) experiments, the presence of VP35 results in increased levels of expression of the cotransfected IRF and, to a variable extent, the cotransfected IKKεKN. The molecular basis of this effect is unclear, but this may influence the efficiency with which VP35 appears to affect kinase-IRF interactions in these different experiments. As previously reported by Cárdenas et al. (7), the amounts of VP35 produced in transfected cells is comparable to what is seen in EBOV-infected cells, suggesting that results obtained using transfected cells are likely to be biologically relevant. Cumulatively, these data demonstrate that VP35 can disrupt the physical interaction between full-length IKKεKN and either IRF-3 or IRF-7, and this physical disruption contributes to the IFN antagonist function of VP35.

The IKKε amino-terminal kinase domain can interact with IRF-3 and with VP35. The amino-terminal kinase domains of IKKε and TBK-1 are quite homologous, with the two proteins exhibiting approximately 70% amino acid identity over their first 350 amino acids (data not shown). To determine if VP35 can interact with the kinase domain of IKKε, a FLAG-tagged IKKεKN deletion mutant consisting of the amino-terminal 315 amino acids (N315) was created (39, 40, 47, 50). This mutant was tested for interactions with either HA-tagged IRF-3 or untagged VP35 by co-IP assay (Fig. 4). Cells were transfected with either full-length IKKεKN (Fig. 4, lanes 1, 3, and 5) or the N315 truncation mutant (lanes 1, 4, and 6) and either HA-tagged IRF-3 (lanes 3 and 4) or VP35 (lanes 5 and 6). HA–IRF-3 and VP35 were expressed in the absence of kinase (Fig. 4, lane 2). Anti-HA and anti-VP35 monoclonal antibodies were then added to the transfected cell lysates for the immunoprecipitation of IRF-3 and VP35, respectively. As shown in Fig. 4, VP35 and IRF-3 each physically interacted with the N315 kinase domain (Fig. 4, lanes 4 and 6) as assessed by Western blotting with anti-FLAG polyclonal antibody, further suggesting that VP35 might physically block the IRF-3–IKKε interaction.

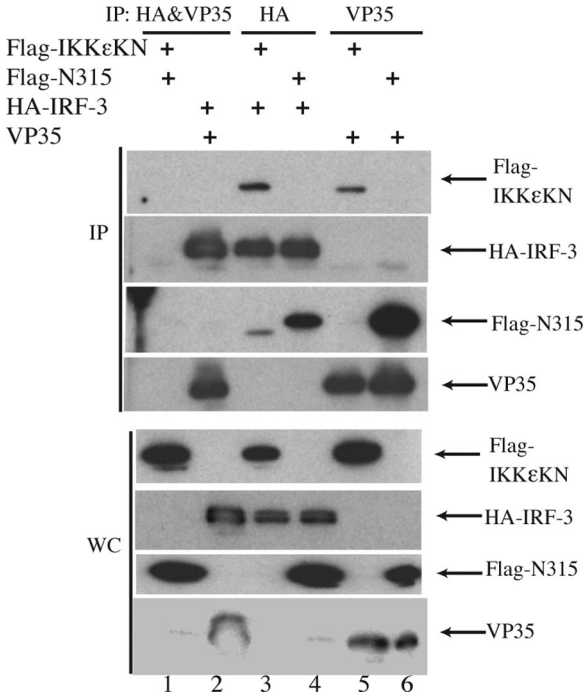

FIG. 4. The kinase domain of IKKε is sufficient for interaction with either IRF-3 or VP35. 293T cells were transfected with expression plasmids encoding the indicated full-length FLAG-tagged IKKεKN (lanes 1, 3, and 5) or the IKKε N315 truncation mutant (lanes 1, 4, and 6) in the absence or the presence of either HA–IRF-3 (lanes 2 to 4) or VP35 (lanes 2, 5, and 6) expression plasmids. Twelve hours posttransfection, cells were infected with SeV. Twelve hours later, cells were harvested and lysed. The precleared lysates were immunoprecipitated (IP) using either monoclonal anti-HA antibody (lanes 1 to 4) or monoclonal VP35 antibody (lanes 1 to 2 and 5 to 6). After SDS-PAGE, Western blotting was performed using anti-FLAG antibody, anti-HA antibody, and anti-VP35 antibody. The expressions of VP35, HA-tagged IRF-3, and FLAG-tagged IKKεKN full-length and N315 deletion constructs were confirmed by Western blot analysis of whole-cell lysates (WC) with anti-VP35, anti-FLAG, and anti-HA antibodies (bottom).

Overexpression of VP35 disrupts IKKεKN N315–IRF-3 interactions. To determine whether VP35 can disrupt binding between the IKKε kinase domain and IRF-3, co-IP experiments were performed using the IKKεKN kinase domain (N315). FLAG-tagged N315 was expressed in 293T cells alone (Fig. 5, lane 1) or with HA–IRF-3 (lanes 3 to 9) in the absence (lane 4) or presence (lanes 6 to 9) of increasing amounts of VP35. VP35 was expressed in the absence of a kinase domain or HA–IRF-3 (Fig. 5, lane 2). IRF-3 was immunoprecipitated with anti-HA antibody, and coprecipitation of the N315 kinase domain was assessed by Western blotting using polyclonal anti-

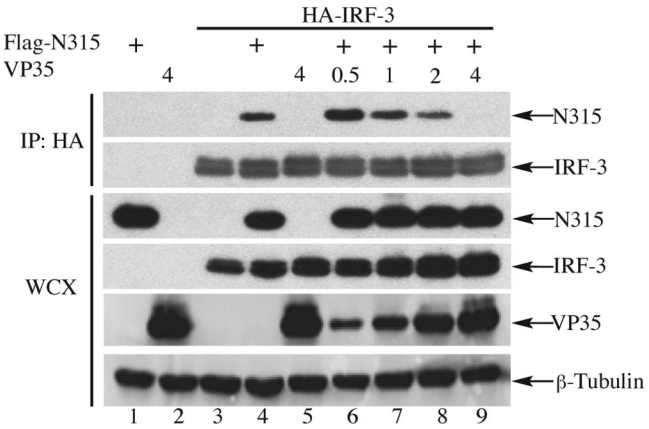

FIG. 5. VP35 disrupts interactions between the IKKε kinase domain and IRF-3. 293T cells were transfected with expression plasmids encoding the indicated proteins (FLAG-N315 [lanes 1, 4, and 6 to 9], VP35 [lanes 2 and 5 to 9], and HA–IRF-3 [lanes 3 to 9]). Twelve hours posttransfection, cells were infected with SeV. Twelve hours later, cells were harvested and lysed. The precleared lysates were immunoprecipitated (IP) using monoclonal anti-HA antibody. After SDS-PAGE, Western blotting was performed using anti-FLAG antibody (Sigma) and anti-HA antibody (Sigma). Expression of VP35, the FLAG-tagged kinase N315 mutant, and HA-tagged IRF-3 constructs was confirmed by Western blot analysis of whole-cell lysates (WCX) with anti-VP35, anti-FLAG, and anti-HA antibodies (bottom).

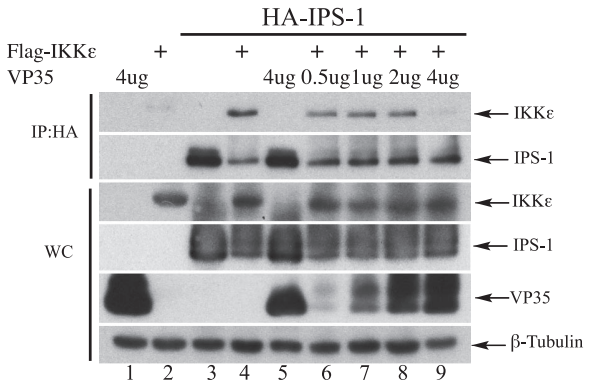

FIG. 6. VP35 disrupts IKKε interactions with IPS-1. FLAG-tagged IKKε, HA–IPS-1, and VP35 expression plasmids were transfected alone (lanes 1 to 3), or HA–IPS-1 was cotransfected with FLAG-IKKε alone (lane 4) or in the presence of increasing amounts of VP35 expression plasmid (lanes 6 to 9). HA–IPS-1 was then precipitated with anti-HA antibody (IP:HA), and the resulting pellets were analyzed by Western blotting with anti-FLAG and anti-HA antibodies. Whole-cell lysates (WC) were analyzed by Western blotting with anti-FLAG, anti-HA, and anti-VP35 antibodies.

FLAG antibody. IRF-3 interacted with N315, but the amount of N315 coprecipitated decreased as levels of VP35 increased (Fig. 5, top). Therefore, the presence of VP35 physically disrupts IKKε kinase domain–IRF-3 interaction in a concentration-dependent manner.

VP35 overexpression disrupts the IKKε–IPS-1 interaction. IKKε also interacts with IPS-1 (33). To determine whether VP35 might influence this interaction as well, we performed co-IP experiments similar to those shown in Fig. 5, using IPS-1 as an IKKε binding partner. Cells were transfected with HA–IPS-1 (Fig. 6, lanes 3 to 9) and FLAG-IKKε (lanes 2, 4, and 6 to 9) expression plasmids in the absence or presence of increasing amounts of VP35 (lanes 5 to 9). HA–IPS-1 was immunoprecipitated by adding anti-HA monoclonal antibody to the cell lysates, and co-immunoprecipitation of FLAG-IKKε was assessed by Western blotting using anti-FLAG polyclonal antibody. The IKKεKN–IPS-1 interaction was impaired, as shown in Fig. 6 (lane 9, top). However, the inhibition did not show a linear dose response to VP35 and was seen only in the samples where the maximum amount of VP35 plasmid was transfected. Cumulatively, these data suggest that the presence of VP35 can disrupt interactions between IKKε and IRF-3, IRF-7 (Fig. 3A and C), and IPS-1 (Fig. 6).

VP35 decreases IRF-3 kinase activity in IKKε-expressing cells. Previous data showed that the presence of VP35 decreased levels of phosphorylated IRF-3 present in cells following SeV infection (2). The data described above suggest that VP35 inhibits the ability of IKKε to interact with IRF-3. To determine whether VP35 expression decreases the ability of IKKε to phosphorylate IRF-3, an in vitro kinase assay was performed using as a source of enzyme lysates from cells transfected with IKKε plasmid in the presence or absence of VP35 plasmid. Cells were transfected with either empty vector (Fig. 7, lane 1) or FLAG-IKKε (lanes 2 to 6) and increasing amounts of VP35 (1, 2, and 4 μg) (lanes 3 to 5) or EBOV NP (4 μg) (lane 6) as an irrelevant protein control. Transfected cell lysates were added to kinase assays where GST–IRF-3-C served as a substrate (Fig. 7, lanes 1 to 6) and were subsequently purified on glutathione beads. The precipitated products were then separated by SDS-PAGE and developed by Coomassie blue staining (Fig. 7, middle) and by autoradiography (top). As

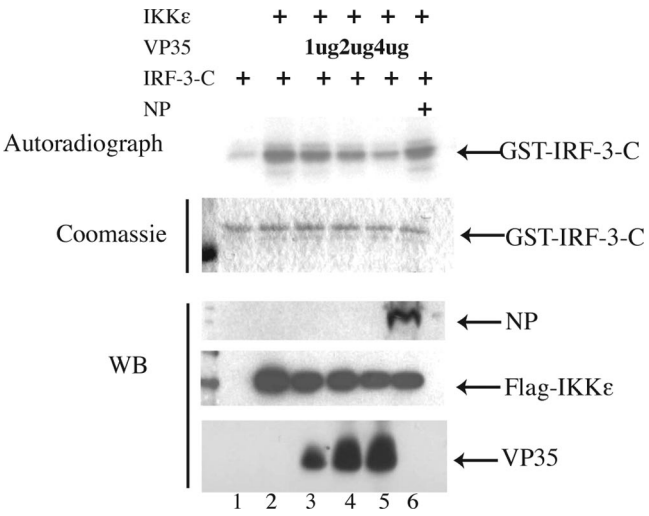

FIG. 7. VP35 decreases IRF-3 kinase activity in IKKε-expressing cells. In vitro kinase assays were performed using GST–IRF-3-C (lanes 1 to 6) as a substrate and lysates from cells cotransfected with IKKε expression plasmid (lanes 2 to 6) and either empty vector (lane 2), increasing concentrations of VP35 expression plasmid (lanes 3 to 5), or NP expression plasmid (lane 6) (VP35 transfection was performed in duplicate). GST–IRF3-C (residues 375 to 427) was then immunoprecipitated using glutathione-Sepharose. (Top) Phosphorylated GST–IRF-3-C as assessed by SDS-PAGE and autoradiography. (Middle) Coomassie blue-stained GST–IRF-3-C included in the kinase assays. (Bottom) Immunoblotting for transfected VP35, IKKε, and NP. WB, Western blot.

shown in Fig. 7, equal amounts of IRF-3 phosphorylation (top), as determined by densitometry, were detectable in samples with IKKε alone or IKKε plus EBOV NP. However, the phosphorylation of IRF-3 decreased in the presence of VP35 to 89%, 79%, and 57% of the control. Levels of GST–IRF-3 (Fig. 7, middle), IKKε, NP, and VP35 are provided for comparison (Western blotting using anti-FLAG, anti-NP, and anti-VP35 monoclonal antibodies are shown in the bottom three panels). Therefore, the presence of VP35 reduces IRF-3 phosphorylation by IKKε.

DISCUSSION

The EBOV VP35 protein antagonizes the IFN-α/β antiviral response (2, 3). This occurs, at least in part, because VP35 inhibits the virus-induced activation of an IFN-β promoter by blocking the activation of IRF-3 (2). This report demonstrates that VP35 can block the activation of an IRF-7-dependent promoter as well. Further studies showed that VP35 can block the activation of the IFN-β promoter induced by the expression of any of several components of the RIG-I/MDA-5 signaling pathway (7). However, VP35 did not detectably inhibit IFN-β promoter activation induced by the expression of a constitutively active IRF-3 (2, 7). These observations suggested that VP35 acts at the level of the IRF kinases TBK-1 and IKKε. This report provides the first evidence that VP35 physically interacts with and is phosphorylated by the cellular kinases IKKε and TBK-1 and suggests that the VP35 interaction with IKKε and TBK-1 contributes to the suppression of IFN-α/β gene expression by EBOV.

We determined that VP35 binds to both IKKε and TBK-1, each of which can activate IRF-3 and IRF-7 in response to RNA virus infection. Several cellular signaling pathways, including Toll-like receptor 3, Toll-like receptor 4, and the RIG-I- and MDA-5-activated pathways, signal through IKKε and/or TBK-1, cellular kinases that phosphorylate IRF-3 and induce IFN-α/β production (10, 20, 32, 38, 46, 49). However, the two kinases are not functionally identical. TBK-1-deficient mouse embryonic fibroblasts have impaired IFN-α/β responses to virus infection, although residual IKKε may partially compensate for the loss of TBK-1. In contrast, TBK-1 was completely dispensable for IFN-α/β responses to virus infection in mouse bone marrow-derived macrophages where IKKε function was predominant (20, 32, 38). Because of this cell type-specific activity, it may benefit EBOV, which productively infects numerous cell types in vivo, to encode a mechanism to target both kinases. Our co-IP data suggest that VP35 may succeed in targeting both kinases by interacting with their kinase domains, which are relatively homologous to one another. The presumed consequence of these observations would be the loss of IFN-α/β production in many cell types. It should be noted, however, that Toll-like receptors 7 and 9 activate IFN-α production through a pathway in which IKKα activates IRF-7 in some cell types, such as plasmacytoid dendritic cells (21). Our data do not determine the impact of VP35 on this alternate source of IFN-α. IKKε also phosphorylates STAT1, affecting the function of the IFN-α/β-activated transcription factor complex ISGF3 (48). It will be of interest to determine whether VP35, via its ability to interact with IKKε, might also affect its ability to phosphorylate STAT1.

The EBOV VP35 protein is functionally equivalent to the phosphoproteins (P proteins) of other members of the *Mononegavirales*, a family that includes rhabdoviruses, paramyxoviruses, and Borna disease virus. Like other P proteins, VP35 plays an essential role in viral RNA synthesis and interacts with the viral nucleoprotein and the viral RNA-dependent RNA polymerase. It is notable, therefore, that the P proteins of rabies virus (a rhabdovirus) and Borna disease virus also target IKKε and/or TBK-1. Additionally, the V proteins of several paramyxoviruses (V proteins are encoded by the P gene and share a common amino-terminal domain with P proteins) also target IKKε and/or TBK-1. For example, the rabies virus P protein blocks IRF-3 phosphorylation by TBK-1, thereby blocking the production of IFN-β (6). Similarly, the Borna disease virus P protein and paramyxovirus V proteins block IRF-3 phosphorylation by acting as alternative substrates for TBK-1 and IKKε (31, 51). Thus, the ability to block IKKε and TBK-1 interactions with and phosphorylation of IRF-3 and/or IRF-7 appears to be a function common to this class of viral proteins.

However, VP35-kinase interactions may have broader effects on the activation of IKKε, as higher levels of VP35 also disrupted IKKε–IPS-1 interactions (Fig. 6). IPS-1 is an upstream binding partner of IKKε and TBK-1 that is important for the activation of these kinases via the RIG-I and MDA-5 pathways and for the production of IFN-β during the antiviral response (10, 20, 25, 32, 33, 45, 46, 52). The capacity of VP35 to target the IKKε–IPS-1 interaction suggests that VP35 may be able to at least partially prevent the activation of IKKε and TBK-1 kinases when high concentrations of VP35 are present.

The observation that VP35 is phosphorylated by IKKε and TBK-1 suggests the possibility that VP35 function may be modulated by these kinases. Consistent with this possibility, metabolic labeling of 293T cells transfected with VP35 plasmid with $^{32}P_i$ resulted in VP35 labeling (data not shown). Whether VP35 becomes phosphorylated in EBOV-infected cells is not certain. However, previous studies demonstrated the phosphorylation of NP and VP30 (4, 8) and demonstrated a functional significance for VP30 phosphorylation (34). The extent to which VP35 may be phosphorylated by IKKε or TBK-1 in EBOV-infected cells will obviously be influenced by the extent to which VP35 prevents kinase activation (e.g., by blocking kinase–IPS-1 interactions) versus the extent to which VP35 serves as a decoy substrate for these kinases.

Recent studies employing recombinant EBOVs highlight the importance of VP35 for suppressing host IFN responses. EBOVs with single-amino-acid substitutions that impair VP35 IFN-antagonist function activated IRF-3 more fully and induced a stronger IFN response, as indicated by a global analysis of host gene expression, than did a parental virus with wild-type VP35 (17, 18). Mutation of VP35 also impaired virus replication in vivo in a nonlethal mouse model of infection (16). The mutations tested in the context of EBOV infection have thus far been demonstrated to impair VP35 dsRNA binding activity (7, 16–18). It will be of interest to determine whether these mutations also impair VP35-IKKε and/or VP35–TBK-1 interactions or whether dsRNA binding and kinase interactions are independent activities of the VP35 protein.

In humans and nonhuman primates, fatal EBOV infections are marked by unchecked viral replication and a lack of an effective antiviral response. In order for the virus to overtake

the host, it must presumably suppress early antiviral innate immune responses. Blocking the phosphorylation of IRF-3 and/or IRF-7 by physically disrupting their interaction with the upstream kinases IKKε and TBK-1 would presumably accomplish this. We and others therefore hypothesized that VP35 will play a critical role in pathogenesis (1, 5). The direct demonstration of a role for VP35 in the development of Ebola hemorrhagic fever awaits testing of VP35 mutant viruses in appropriate animal models using VP35 mutant viruses. However, the data in this report showing that VP35 targets IKKε and TBK-1 shed light on at least one of the mechanisms of IFN antagonism by VP35. Further studies of this protein suggest novel vaccine or antiviral strategies.

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